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Antibody Production in the Goat: Immunokinetics and Epitope Specificity Using a Glycoprotein Immunogen

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ABSTRACT—We have developed methods for the efficient production of antibodies in goats using a glycoprotein purified from the eggs of the frog *Xenopus laevis*. When emulsified with complete Freund's adjuvant, the amount of immunogen required to elicit high titer antiserum was much less than commonly used (50 μg as opposed to mg quantities). A single immunizing injection produced a maximum antibody response and secondary immunizations were not beneficial. The IgG was directed toward the carbohydrate moiety when the glycoprotein was used as immunogen, although the protein moiety was immunogenic as shown by the use of the deglycosylated polypeptide. Goats immunized with glycosylated immunogen all produced maximum titer antisera from 35 to 45 days postimmunization. In contrast, maximum titer in goats injected with deglycosylated polypeptide was reached at 26 days postimmunization. We anticipate that the immunization methods reported here will improve the usefulness of the goat for preparing polyclonal antibodies.

INTRODUCTION

Methods for producing polyclonal antibodies in laboratory rodents, such as mice and rabbits, are experimentally well defined and commonly used. Although goats are particularly useful for the production of antibodies because large amounts of antiserum can easily be collected, experimentally determined methods for immunization have not been published. Immunizing procedures for goats and sheep are usually extrapolated from those designed for laboratory rodents. For example, it has been stated that the amount of antigen necessary to stimulate production of antibodies is approximately proportional to body weight. That is, if 100 μg of protein is optimal for a rabbit, 5–10 mg is required for a goat [1]. Secondary immunization (boosting) is also thought to be necessary to produce maximum titer antisera. Although these extrapolated methods are currently used [2–4], there is an acknowledged need for a systematic study of the immune response in the goat [1, 5].

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The purpose of the research reported here was to define methods for the most efficient production of polyclonal antibodies in goats for our purposes i.e. the minimal amount of immunogen required to produce a maximal antibody response in a minimum amount of time using a single immunizing injection. We used as our immunogen a presumably antigenically foreign purified glycoprotein, the *Xenopus laevis* cortical granule lectin. The time required to produce maximum IgG and IgM titers as a function of immunization dose and effects of secondary immunization on titer were determined. We also characterized the epitope specificity of the antibodies produced to the glycosylated and deglycosylated forms of the lectin and their macromolecular and tissue specificity.

MATERIALS AND METHODS

Antigen preparation

Xenopus laevis cortical granule lectin (CGL) was purified from eggs as described [6]. Trifluoromethane sulfonic acid was used to deglycosylate the CGL [7]. *Xenopus laevis* egg envelopes were prepared as previously described [8].

Protein concentrations were determined using the bicinchoninic acid method with bovine serum albumin as a standard [9].

Immunization procedure

The dairy goats used were does or wethers of the French Alpine and Nubian breeds or cross breds. Goats' ages were variable, ranging from less than one year to 10. No tissue necrosis was observed in any of the animals at the sites used for primary or secondary immunizations.

After dissolving the antigen in 1 ml Tris buffered saline (10 mM Tris-Cl, 150 mM NaCl, pH 7.5), an emulsion was made with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit MI). Double hubbed needles attached to glass syringes were used to make the emulsion as described by Herbert [10]. One intramuscular injection was made between the clavicle and the base of the neck. The first boost immunization, if made, was prepared similarly except that incomplete Freund's adjuvant was used. These boost injections were generally given in the opposite side of the neck. Immunogen for the second boost injection was dissolved in 10 mM phosphate buffered saline, pH 7.4 and injected subcutaneously.

Blood collection and titer determination

Blood was collected by jugular venipuncture into evacuated untreated tubes (Vacutainer, Rutherford NJ) and stored at room temperature for approximately 6 hr to allow clot formation. After refrigeration overnight at 4°C, the clotted blood was centrifuged at 10,000×*g* for 30 min, the serum decanted and frozen at −20°C until use. For large scale collection of blood, we routinely collected 250 ml and typically recovered 65% of the volume as serum.

A kinetic ELISA (enzyme-linked immunosorbant assay) was used to determine the titer of serum samples. Disposable ELISA plates ('Easy Wash', Corning Glass Works, Corning NY) were coated overnight at 4°C with 15 ng of antigen in 50 μl of buffer (100 mM Na_2CO_3, pH 9.5). After washing with TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20), 100 μl of serial 1:3 dilutions of serum (with TBST) were applied to the wells. For IgM titer determination, IgG was removed from serum samples using a protein G kit (Quik-Sep IgM, Isolab Inc., Akron OH). After incubation at room temperature for 1 hr, plates were washed and 100 μl of a horseradish peroxidase conjugated secondary antibody was added for 1 hr. Either rabbit anti-goat IgG (Fc) or rabbit anti-sheep IgM (μ chain) (both from Organon Teknika Corporation, West Chester PA) was used as the secondary antibody. Plates were developed by the addition of substrate [50 mM citric acid, pH 4.0, 0.60 mM 2,2′-azino bis(3-ethylbenzthiazoline-6-sulfonic acid), 0.08% v/v H_2O_2] and the absorbance at 405 nm was measured every 8 sec for 2 min using a kinetic microplate reader (Molecular Devices, Menlo Park CA). The velocity, ΔA_{405}/minute, was plotted against serum dilution. We defined the antibody titer of antiserum as the inverse of the serum dilution corresponding to half maximal velocity.

To confirm that the reported titers represent immunologically specific binding, a number of controls were tested: 1) In the absence of antigen, insignificant amounts of primary antiserum bound to the ELISA wells. 2) The secondary antisera was tested for nonspecific binding both to the antigen and to the ELISA plate. 3) Finally, coincubation of purified soluble CGL with anti-CGL serum abolished serum binding to the CGL immobilized on the ELISA plate.

Preparation of tissue extracts and analysis of serum specificity

Aqueous extracts were made from fresh *Xenopus laevis* or mouse tissue by crushing diced organs between two frosted glass microscope slides in distilled water. Cellular debris was removed by low speed centrifugtion. The proteins in the supernatant solution were separated by reduced SDS-PAGE [11] and electroblotted onto nitrocellulose [12]. Membranes were probed with antiserum essentially as described for an ELISA, except that the developing solution contained 50 ml 10 mM Tris, 150 mM NaCl pH 7.4, 30 mg 4-Chloro-1-Napthol/10 ml methanol and 50 μl 50% (w/w) H_2O_2.

RESULTS

Immunokinetics of IgG production as a function of immunogen dose

To investigate the minimum amount of immunogen necessary to stimulate maximum IgG production, goats were immunized with 10 to 1000 μg of CGL. Blood samples were collected twice weekly and the IgG titer of the serum determined. Immunization with 50, 100 or 1000 μg of CGL gave virtually identical immune responses. IgG production began 3 to 8 days postimmunization and continued to increase until the maximum titer was reached 35 to 45 days postimmunization. Figure 1 illustrates the immune response of the goat immunized with 50 μg CGL. Maximum titer values ranged from 30,000 to 60,000 titer units. In contrast, immunization with 10 μg of CGL led to a protracted immune response which peaked at a lower titer. For example, on day 42 postimmunization, the IgG titer from the three goats immunized with at least 50 μg CGL was at the maximum, whereas the titer of the goat immunized with 10 μg was around 20% of maximum and reached a maximum of 7000 titer units on day 65.

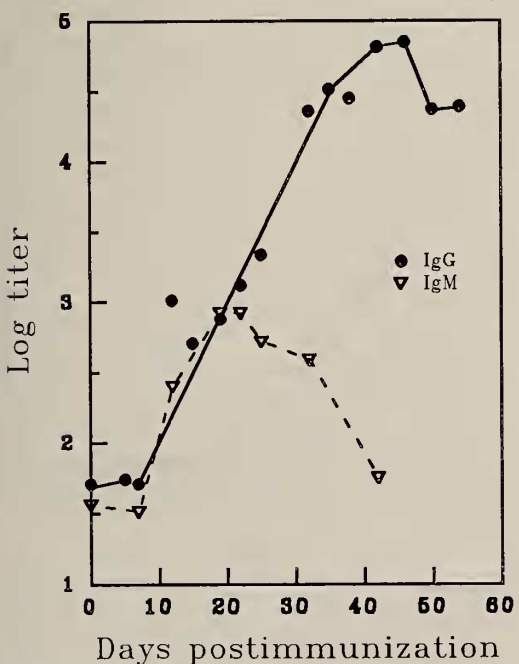

FIG. 1. IgG and IgM response after injection of 50 μg CGL.

Immunization with 100 or 1000 μg of deglycosylated CGL elicited an immune response of slightly different kinetics compared to CGL. These two goats' immune responses were essentially identical, producing maximum titer antiserum 26 days postimmunization (approximately 14 days earlier than goats immunized with 50 μg or more of CGL). In addition, maximum titer values were lower (4,500–6,000 titer units) than those from goats immunized with CGL.

Effect of secondary immunization on IgG titer

The two goats initially immunized with 100 or 1000 μg CGL were given boost injections in an attempt to elicit production of higher titer antisera than was produced after primary immunization. At the time of boosting (273 days postimmunization), the IgG titer had declined to 3% or 7% (respectively) of the maximum from primary immunization. These animals were boosted on two occasions with a total of 80 μg CGL. The first injection was given intramuscularly in an emulsion of incomplete Freund's adjuvant and the second was injected subcutaneously as a solution. After each boost injection, the serum titer increased and reached a plateau, but did not exceed the titer reached after primary immunization (Fig. 2).

Immunokinetics of IgM production

Given the differences in time to reach maximum IgG titer upon injection of glycosylated and deglycosylated immunogen, we looked for similar differences in the kinetics of IgM production. IgG depleted samples from the goat immunized with 50 μg CGL were analyzed for IgM content using an IgM specific secondary antibody. Serum samples from goats immunized with 100 or 1000 μg of deglycosylated CGL were similarly analyzed for IgM content, but without removing IgG.

The IgM titer of the goat immunized with 50 μg CGL was detectable 8 days postimmunization and peaked between 19 and 22 days postimmunization. After day 22, the IgM titer slowly decreased reaching basal levels about 42 days postimmunization (Fig. 1).

The IgM response of both goats immunized with

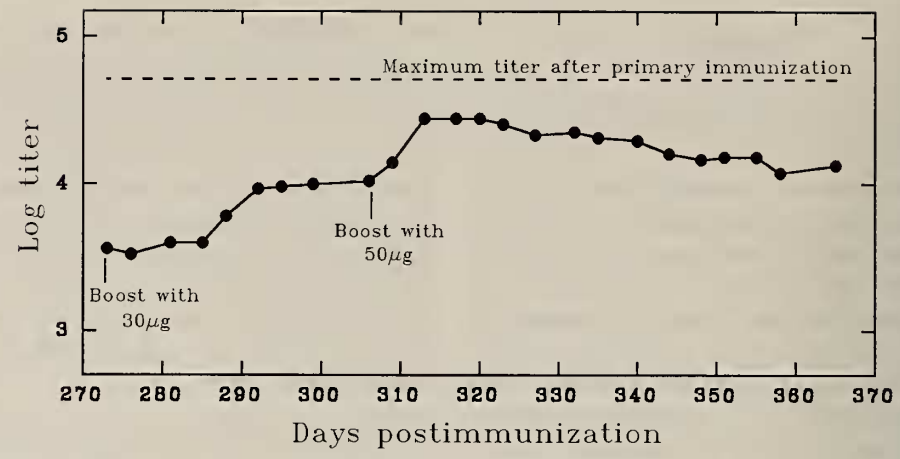

FIG. 2. IgG response after secondary immunization. Goat initially immunized with 100 μg CGL. Prior to boost, titer was 3% of maximum produced following primary immunization.

deglycosylated CGL was initially similar to the goat injected with 50 μg CGL in that IgM appeared 8 days postimmunization. The IgM response of the goat immunized with 1000 μg of deglycosylated CGL, however, seemed accelerated because the IgM titer peaked between 12 and 15 days postimmunization and returned to basal level 22 days after injection. In contrast, the goat injected with 100 μg of deglycosylated CGL exhibited kinetics similar to those of the goat immunized with 50 μg of CGL in that maximum IgM antiserum was produced on day 19 postimmunization and the IgM serum levels declined gradually.

Epitope specificity of antibodies generated to CGL and deglycosylated CGL

Upon testing the macromolecular and tissue specificity of antisera, we observed marked cross-reactivity of CGL antiserum with *Xenopus laevis* egg envelope glycoproteins. However, the CGL antiserum was tissue specific as it did not react with aqueous extracts made from *Xenopus laevis* skeletal muscle, liver, gall bladder, spleen, lung, nerve, and activated egg cytosol. In addition, it did not react with extracts from mouse lung, kidney, liver, heart, or skelctal muscle (data not shown). This cross reactivity with egg envelope glycoproteins prompted an investigation of whether carbohydrate or protein domains were recognized by the CGL antibody. When CGL was used as an immunogen, approximately 96% of the antiserum reactivity was directed against the carbohydrate moiety (Fig. 3). In addition virtually all crossreac-

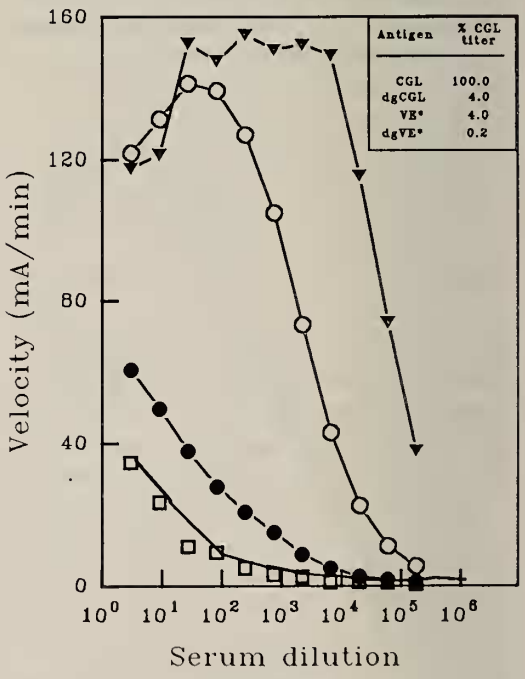

FIG. 3. ELISA reactivity of CGL antiserum. Reactivity of anti-CGL serum with given antigens. ▼ CGL; ▽ VE*, vitelline egg envelopes derived from activated, dejellied eggs; ● dgVE*, deglycosylated vitelline egg envelopes; □ Preimmune serum; dgCGL, deglycosylated CGL.

tivity with egg envelope glycoproteins was due to shared carbohydrate residues, as deglycosylation rendered envelope glycoproteins unreactive to the CGL antiserum (Fig. 3). The deglycosylated CGL antiserum showed no significant crossreactivity with egg envelope glycoproteins, thereby indicating that the CGL and envelope protein moieties were non-homologous (as expected from the limited amount of amino acid sequence data available on these glycoproteins [13, 14, H. Fabry, M. Oda, and J. Hedrick, unpublished observations]).

DISCUSSION

Although goats are commonly used to produce commercial secondary antisera, rabbits and mice are used more often for production of primary antisera both in the research laboratory as well as for commerce. Part of this preference likely stems from the belief that milligram quantities of antigen are required for immunization of larger animals. From our observations, only small amounts of immunogen are required for the goat. We observed no differences in the kinetics or magnitude of the IgG response when goats were immunized with as little as 50 μg or as much as 1000 μg of CGL. These goats all produced maximum titer antisera (30,000 to 60,000 titer units) 35 to 45 days postimmunization. Although the IgG response after immunization with 10 μg CGL was markedly slower, useful antisera was eventually produced (7000 titer units, 65 days postimmunization).

Most immunization schedules call for boost injections at intervals after the primary immunization to increase antiserum titer. In addition, it is often recommended to delay boosting until the titer in response to primary immunization has declined to a small percentage of maximum. Our observations were contrary to this common practice. We boosted goats initially injected with 100 μg and 1000 μg CGL on two occasions with a total of 80 μg of CGL. After each boost, serum titers increased and reached a plateau, but at no time was the post boost titer significantly higher than that produced after primary immunization. Thus, when adequate amounts of immunogen are used for the primary immunization using complete Freund's adjuvant in the goat, boosting is not required for generation of maximum titer antiserum. However, if relatively small amounts of immunogen are used (<50 μg), boost injections may aid in increasing antibody production.

When goats were immunized with CGL, the dominant immune response was directed against the carbohydrate or oligosaccharide moiety of the glycoprotein. Characterization of the sugar moieties recognized by the CGL antiserum is currently being investigated and preliminary results suggest that a modified sialic acid may be the immunodominant monosaccharide present on the oligosaccharide sidechains of CGL (H. Fabry, N. Wardrip, and J. Hedrick, unpublished observations).

Since the presence of highly immunogenic carbohydrate structures on the lectin might lead to an atypical immune response, we immunized additional goats with 100 μg and 1000 μg of deglycosylated CGL. The kinetics and magnitude of IgG production by these goats was not dose dependent, similar to what was observed when glycosylated CGL was used. However, the immune response was accelerated and of lower magnitude than the goats immunized with the glycosylated lectin. These differences may reflect genetic variance between animals or result from the relative immunogenecity of the carbohydrate versus the protein moieties of the CGL.

We have used more than 15 goats in our studies and all have produced strong immunological responses to small quantities of *Xenopus laevis* and *Sous scrofa* (pig) protein as well as glycoprotein immunogens. We feel, therefore, the methods described in this paper can be successfully applied to a variety of immunogens, and will improve the usefulness of the goat for production of primary antisera.

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